Concentration-Temperature Superposition of Helix Folding Rates in Gelatin

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We study the kinetics of helix-coil transition in water solutions of gelatin (collagen protein) by optical rotation techniques combined with thermal characterization. By examining the rates of secondary helix folding, and covering a very wide range of solution concentrations, we are able to identify a universal exponential dependence of folding rate on concentration and quench temperature. We demonstrate a new concentration-temperature superposition of data at all temperatures and concentrations, and build the corresponding master curve. The results support the concept of a diffuse helix-coil transition. We find no concentration dependance of the normalized rate constant, suggesting first order (single) kinetics of secondary helix folding dominate in the early stages of renaturation.

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Kinetics of protein folding is one of the cornerstone problems in understanding the full mystery of biologically active macromolecules [1, 2]. A number of experimental techniques focus on a great variety of natural and synthetic polypeptides undergoing their globular collapse, or denaturation into the coil. Although one might want to think of such a transition in terms of individual macromolecules, there is much evidence that cooperative effects between different chains play a significant role, an effect often referred to as oligomerization [3]. This extreme variability has, to date, defied any attempt on universal description, beyond the classical Zimm-Bragg abstraction [4].

In this Letter we examine perhaps the most well-studied protein, collagen, and discover that the complexity of its folding kinetics, strongly dependent on temperature and concentration in the solvent, can be dramatically simplified. All of this kinetics can be scaled onto a single master curve by a new procedure we call the "concentration-temperature superposition". There is a remarkable analogy: the classical time-temperature superposition [5, 6] has allowed master curves to describe the glass transition in a variety of thermal viscoelastic systems and served a great purpose in rheology for the last 50 years. A much more recent discovery of time-concentration superposition [7, 8] has allowed the universal description of dynamic glass, or jamming transition, in lyotropic systems such as colloid suspensions. In both cases the cooperativity of interparticle interaction is the key, and we shall argue that it is relevant for the folding kinetics as well.

We must emphasize that we study the principal helix-coil transition in collagen chains, i.e. the folding of secondary helices. This is achieved by our experimental methods focusing on optical rotation of linearly polarized light at a wavelength away from any absorbtion band. It has been recently demonstrated that the dominant contribution to the measured rotation rate arises from the secondary helices [9]. This is different from the more common studies of gelation kinetics in gelatin, which is controlled by the tertiary triple helices and is usually studied by rheological methods. The field of sol-gel transition and mechanical response of networks is broad and well-established [10, 11]. Recent important observations [12] of universal master curves, describing specifically the storage modulus in gelatin, also make connections with glassy dynamics. We shall see from the results below that our present work is not related to these ideas: we examine much shorter times at which the secondary structure is formed, while the kinetics of subsequent rheological is a much slower process.

Collagen is the main protein component of white fibrous connective tissues such as skin, tendons and bones. The fundamental unit of the native collagen structure is a rod consisting of three individual molecular strands, each twisted into a secondary left-handed helix, which analogous and very similar to the classical α-helix in other polypeptides. In collagen, three segments of secondary helix wrap together to form a tertiary right-handed superhelix stabilized by further interchain hydrogen bonding [13]. Collagen is extracted from tissues by hydrolytic degradation, which denatures it producing the resulting material commonly known as gelatin.

Gelatin is dissolved in water by heating the solution to $40°C$. Above this temperature collagen chains have random coil configuration. On cooling transparent gels form containing extended physical crosslinks. X-ray diffraction and transmission electron microscopy measurements suggest that the crosslinks are formed by partial reversion to ordered triple-helical segments, separated along the chain contour by peptide residues still in the random coil configuration [14, 15]. At very low concentrations the renaturation, or folding process is completely intramolecular and proceeds by a back folding of the single chains [16]. With increasing concentration the renaturation becomes increasingly intermolecular. Gelation occurs at concentrations above $5\,mg/ml$ due to the formation of an infinite elastic network in the gelatin solution, see [12, 17] and references therein.

Gelatin owes many of its uses to this coil-helix transition. The kinetics of the transition has been extensively studied for many years using a variety of tech-

niques such as differential scanning calorimetry (DSC), scattering and rheometry, as well as optical rotation. Figure 1(a) shows a sequence of DSC traces of helix-coil transition that we obtained in gelatin solutions of different concentration, while Fig. 1(b) shows the corresponding transition line, $T_m(c)$. A very flat dependence of the transition temperature on concentration in the thermal phase diagram, extrapolating to $c \rightarrow 0$, indicates that the transition is largely intrachain. Note that the gelation phase diagram, obtained by rheological methods, would show a drop of the gel point $T_g(c)$ below 5 mg/ml. Also note that, since the melting temperature depends strongly on the gelation temperature and time, a broad range of $T_m(c)$ values are found in the literature.

Gelatin, as any protein, is an optically active material in both the random coil and helical states. However, due to coherent long-range chiral ordering, helical domains rotate the plane of light polarization much more strongly than the individual chiral aminoacids in the coil state. Thus, the coherent optical activity gives a direct indication of the fraction of the monomers in helical states.

In many biopolymers, including polysaccharides and DNA, the coil-helix transition is very fast and resembles a true first order phase transition [20]. In gelatin, however, the helix nucleation step lasts several minutes and subsequent growth of the helices proceeds even slower, at a logarithmic rate [21]. This allows one to study its detailed kinetics. Most investigations have focused on isothermal renaturation rather than temperature scanning studies, because of the difficulty in accessing the equilibrium state. This slowness of the process will allow us to scan and superpose the renaturation kinetics over a range of temperatures and concentrations.

In one of the earlier studies, Flory and Weaver studied helix growth rates in very dilute gelatin solutions ($c < 4$ mg/ml) and found first order kinetics [22], i.e. that the folding rate is concentration-independent. They postulated the coil-helix transition proceeds via an intermediate state formed by intramolecular rearrangement of a single chain. Assuming this state consists of a secondary

helix segment, consideration of the minimum stable segment length leads to the Flory-Weaver expression for the rate constant for the renaturation after quenching the dilute solution:

$$k_1 = B \exp\left(\frac{-A}{kT\Delta T}\right), \qquad (1)$$

where A and B are constants, T is the quench temperature, and $\Delta T = T_m - T$ is the degree of supercooling below the equilibrium melting temperature T_m.

The first order kinetic analysis agrees with other experimental observations of dilute gelatin solutions [16]. In semidilute solutions concentration-dependant kinetics has been observed [17, 23]. Consequently, new mechanisms have been proposed, involving either different segments of the same chain or up to three different peptide chains having to interact to form stable helices. The topological consequences of these arrangements on helix formation and gelation are very significant.

Goddard et $al.$ [18], suggesting an analogy between renaturation and crystallization, modelled the kinetics in terms of Avrami exponents [24]: $\chi = 1 - \exp[-k_1 t^n]$, where χ is the helix fraction. They obtained the exponent n close to unity, suggesting one-dimensional growth from predetermined nuclei and supporting the first order kinetics ideas. Early optical rotation studies confirmed that simple exponential kinetic occurs in dilute solutions, but only in the initial stages of renaturation [25, 26]. Most recently, Guo et $al.$ [17] studied initial renaturation rates of gelatin solutions at semidilute concentrations up to 0.12 g/ml and observed what appeared as a combination of first order and second order kinetics, i.e. the rate of growth of the normalized helix fraction had a linear concentration dependence. This seems unusual, in view of the triple nature of tertiary helix linkages in collagen. However, the observations of second-order gelation kinetics, in particular the elastic modulus proportional to c^2, have been made for over a century [27]. A two-step mechanism with the rate limiting step formation of a nucleus of two helices wrapped together, followed by rapid subsequent wrapping of another coil segment to form the triple helix has been proposed a long time ago to account for these findings [12, 28]. However, we shall argue that there is a big difference between optical rotation and rheological studies. The latter return the viscoelastic response of the gel and thus rely on the triple-helix linkages of the network. Our opinion [9] is that the properly measured optical rotation signal is determined by secondary helices and not the tertiary structure. We attribute the second-order kinetics of Guo et $al.$ [17] to the range of examined concentrations - only up to ~ 0.1 g/ml (which is also the range in the rheological work of Normand et $al.$ [12]). Overall, examining the literature of the last 30 years, one finds no universality, nor agreement between different concepts.

In an attempt to link together all of the past findings, we studied the helix-coil transition in gelatin over a much wider concentration range, up to 0.4 g/ml. The universal
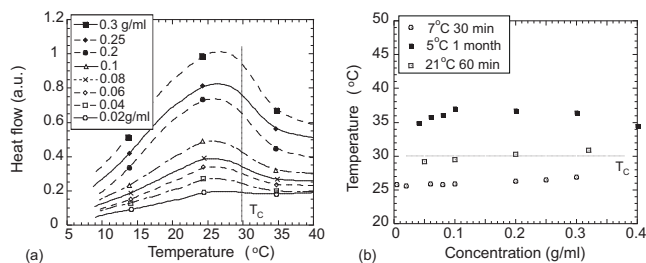

FIG. 1: (a) DSC traces demonstrating the helix-coil transition in gelatin/water solutions of different concentrations, labelled on plot. (b) A thermal phase diagram of this transition, $T_m(c)$, obtained from the DSC data ($\odot$), with results for different preparation methods from the literature ($\square$ - [18], $\blacksquare$ - [19]). The value T_c is obtained from the concentration-temperature superposition analysis below.
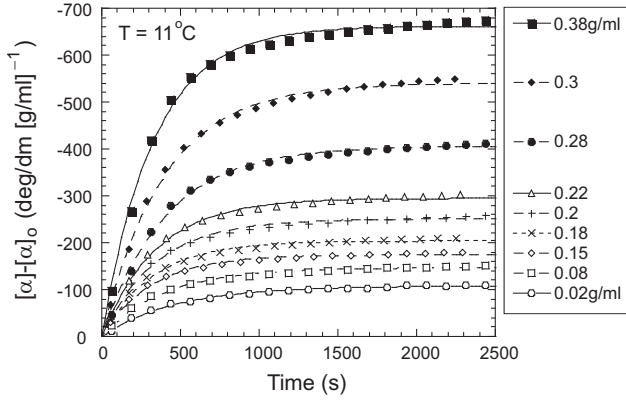
FIG. 2: Typical traces of normalized optical rotation, directly proportional to the secondary helix density, as function of time after quenching the denatured collagen solution to $T_Q = 11°C$. Different data sets correspond to concentrations labelled on graph. Solid/dashed lines are fits with $A(1 - \exp[-t/\tau])$.

FIG. 3: Initial rates of helical nuclei growth, R_0 at $t \to 0$, as functions of solution concentration, for a range of quench temperatures labeled on plot. Solid/dashed lines are fits with exponential $B \exp[Y c]$. The arrow indicates the concentration range of earlier studies [17].

FIG. 4: The master curve of growth rate vs. concentration, $R_0(\widetilde{c})$, superposing the results for different quench temperatures (labelled on plot). The arrows point at different data sets; the solid line is a universal fit with $B \exp[Y \widetilde{c}]$.

master curves we construct from the new superposition procedure give explicit predictions for $c \approx 0.65$ g/ml, and can be further extended to the full range of temperatures and concentrations. The high-sensitivity differential optical rotation detector we used to measure the secondary helix content has been described in, e.g., [29]. The raw measurement of the total angle of polarization plane rotation, Ψ, is divided by the (constant) sample thickness to produce the rotation rate. It is then normalized by the solution concentration to obtain a specific rotation $[\alpha] = (1/c)[d\Psi/dz]$, from which we subtract a bare value $[\alpha]_0$ corresponding to the average aminoacid optical activity, separately measured in the coil state at high temperatures. The resulting difference is proportional to the concentration of correlated helices in the medium. The typical reading, for a range of concentrations, is shown in Fig. 2 for a solution quenched from 50°C to the value $T = 11°C$ in this case. As a result of $[\alpha]$ normalization, the y-axis in Fig. 2 is directly proportional to the helix fraction in the sample.

There are important and delicate issues of the slow drift of collagen towards its natural state [21], reflected in the small deviation of the long-time data from the simple exponential fit of each data set (shown by the lines in Fig. 2). For instance, the rheological study of Normand *et al.* [12] is heavily based on this regime, where the tertiary structure (and the gel elasticity) are being consolidated and the elastic modulus increases dramatically. We, however, are concerned with the initial rates of secondary helix growth, $R_0(c, T)$, essentially the slopes $R_0 \equiv d[\alpha]/dt = A/\tau$ at $t \to 0$, from the fitted functions $A[1 - \exp(-t/\tau)]$ in Fig. 2. For each quench temperature, these slopes depend on the solution concentration. The summary of this dependence is given in Fig. 3. The highly non-linear concentration dependence of growth rates is apparent from the exponential fits of all data sets. We note that the highest solution concen-
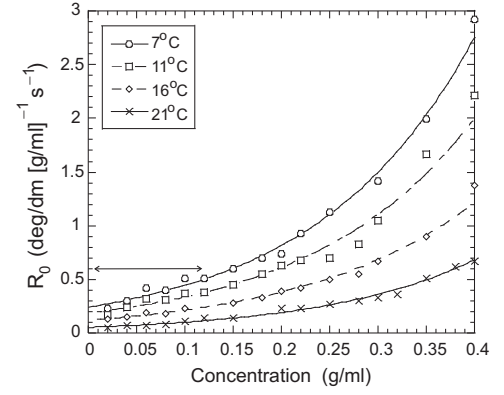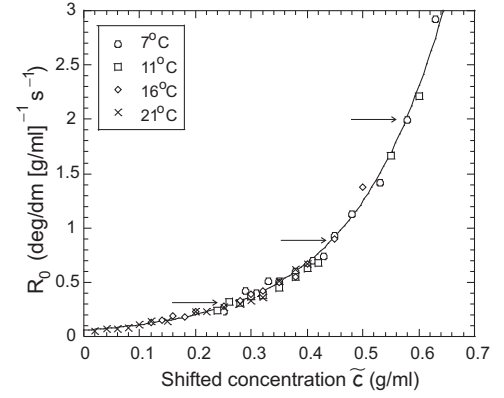
tration quantitatively studied so far was $c \sim 0.12$ g/ml, by Guo *et al.*[17].

Guided by the experience in time-temperature and time-concentration superposition, we notice that the sets of data for the growth rates in Fig. 3 can be shifted along the concentration axis by an amount that is a function of quench temperature. Selecting a reference temperature (at this stage, we arbitrarily choose $T_{ref} = 21°C$) one scales the concentration c for each data set such that $\widetilde{c} = \beta \cdot c$, with the coefficient (the shift factor) a function of the quench temperature for each data set, $\beta = \beta(T)$. The fact that these sets do superpose means that there is a universal underlying expression for the growth rate $R_0[\beta(T)c]$. The resulting master curve, Fig. 4, is an indication of such a universal physical process that controls the helical nucleation and growth in all regions of the (T, c)-phase diagram.

It is important to establish a law of the shift factors β dependence on temperature. Figure 5 gives the plot
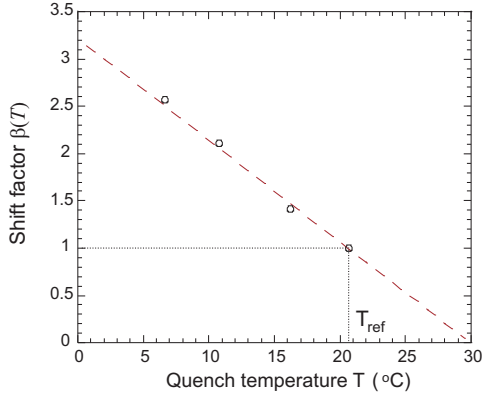
FIG. 5: Dependence of the shift factor β on quench temperature T_Q. The reference temperature chosen at 21°C corresponds to the original, non-shifted data – or $\beta = 1$. The linear fit (dashed line) crosses the $\beta = 0$ line at ~ 30°C.

FIG. 6: Normalized folding rates k as functions of concentration at different quench temperatures (labelled on plot).

of a set $\beta(T)$ required to produce the superposed master curve for the initial growth rate. It follows quite an obvious linear function, $\beta = 0.1(T_c - T)$, with the "critical temperature" $T_c \approx 30$°C (more precisely, 302.5°K) defined as the point at which $\beta = 0$. This temperature is labelled in the gelatin phase diagram and the sequence of DSC scans, Fig. 1(a). Clearly, its relation to the transition line is not accidental. In fact we should conclude that (within errors of our experiment and analysis) this critical temperature is indeed the line of the helix-coil transition, $T_c = T_m(c)$. With this assumption, the helical growth rate, as obtained from the master curve in Fig. 5, follows the equation

$$\left. \frac{d[\alpha]}{dt} \right|_{t \to 0} = B \exp\left[b(T_m - T)c \right] \qquad (2)$$

with fixed parameters $B \approx 0.06 \, \text{deg/dm} \, (\text{g/ml})^{-1}\text{s}^{-1}$ and $b \approx 0.6 \, (\text{ml/g})/$°K. Note that we find no critical or singular behavior near the transition. The growth rate becomes exponentially small at $T > T_m$. In retrospect, it is perhaps not surprising that we did not obtain a critical vanishing of the growth rate, as would be the case with classical phase transitions, or was suggested by the old Eq.(1). Our present finding supports the idea of a diffuse nature of helix-coil transformation in a single chain, with chain fluctuations capable of creating a non-vanishing helical fraction above $T_m(c)$ and disrupt thermodynamically equilibrium helices formed below this temperature. In other words, there is no sharp phase boundary $T_m(c)$ for the helix-coil transition. In equilibrium models of this transition one identifies T_m as a point of steepest gradient in helical fraction $\chi(T)$, while in our study of kinetics we obtain roughly the same value at a point when $R_0(c, T)$ changes from the decaying to the growing exponential function $\exp[Yc]$.

Having established the universal master curve for the folding rates $R_0(c, T)$, we must now make contact with the other kinetic studies, that traditionally focus on the
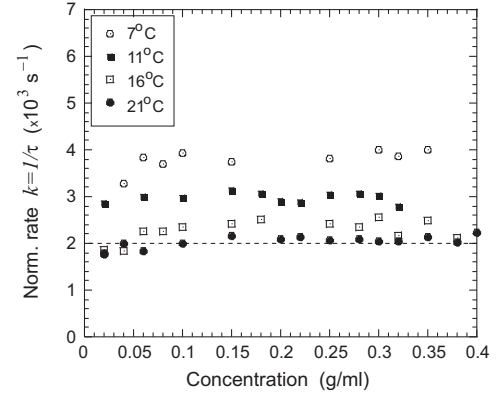
normalized helical fraction, in our notation defined as

$$\chi(t) = \frac{[\alpha] - [\alpha]_0}{A(c, T)} = 1 - \exp(-t/\tau) , \qquad (3)$$

according to Fig. 2 and earlier work [18]. The origin of the exponential concentration dependence, Eq. (2), is in the constant $A(c)$, while the normalized rate $k = 1/\tau \equiv R_0/A$. The concentration dependence of this rate is a signature of process topology, e.g, a constant value means first-order (single-molecule) folding process. Guo et al. [17] claimed a second-order process, i.e. a linear dependence $k = k_1 + k_2 c + ...$, but we clearly arrive at different conclusions. Figure 6 shows our results for the normalized rates k over a concentration range much wider than in any previous work. There are some delicate issues of fitting the data that includes the slow long-time tail, which certainly accounts for the large noise in the $k(c)$ data. However, the qualitative picture clearly suggests that there is no relevant concentration dependence and, therefore, the single-chain folding process (first-order) k_1 is in fact dominant at early stages of renaturation. This may appear a surprising result as, with increasing concentration, intramolecular rearrangements of single chains become limited. Also, the triple-helical nature of collagen helices is well established indeed [27]. However, let us remember that our experimental method inherently looks at the secondary helices (the signal from triple helical structures is very low due to the large wavelength mismatch). The observed first-order (i.e. single-chain) nature of the principal folding process appears to be an indication that the transition from coil to secondary helix is a single chain process, while the association of these helices to form the triple network linkages occurs subsequently and contributes less to the helical onset than was previously thought.

In conclusion, by applying a new concentration-temperature superposition we found a universal master curve describing the initial folding rates R_0 of gelatin solutions over a broad range of concentrations and temperatures, spanning the nominal helix-coil transition point

T_m. The exponential (c, T)-dependence of R_0 arises from the increasing overall amount of helices in different systems. In contrast, the normalized helical fraction $\chi(t)$ grows with a c-independent rate, suggesting the first-order kinetics. One needs to study other oligomerizing proteins to test the further universality of the discovered master curves.

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